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**Title: Impact of natural and artificial prenatal stimulations on the behavioural profile of Japanese quail.**

**Running title:** Prenatal stimulation on bird development

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**Keywords:** auditory stimulation; behavioural development; emotivity; social behaviour; prenatal stress

**SUMMARY STATEMENT**

This article presents the effects of prenatal auditory stimulation of quail embryos. It shows that juvenile quail develop differently depending on the type of stimulation perceived.

**ABSTRACT:**

As the sensory systems of vertebrates develops prenatally, embryos perceive many environmental stimuli that can influence the ontogeny of their behaviour. Whether the nature and intensity of prenatal stimuli affect differently this ontogeny remains to be investigated. In this context, this study aimed to analyse the effects of prenatal auditory stimulations (natural stimulations “NS”: predator vocalisations, or artificial stimulations “AS”: metallic sounds) on the subsequent behaviour of young Japanese quail (*Coturnix coturnix japonica*). For that, behavioural variables recorded during ethological tests evaluating emotional and social reactivity were analysed using a principal component analysis. This analysis revealed significant differences between the behavioural profile of stimulated chicks and that of non-exposed chicks. Indeed, chicks exposed to NS expressed more intense emotional responses in fearful situations, but less neophobia in the presence of a novel environment or object, whereas chicks exposed to AS appeared more sensitive to social isolation. Our original results show that the acoustic environment of embryos can influence the way young birds subsequently interact with their social and physical environment after hatching, and face challenges in changing living conditions.

## Introduction

Sensory systems begin to develop during the prenatal period. In vertebrate and some invertebrate species, it follows a chronological and invariant sequence: the somatosensory system (tactile and vestibular) develops first, followed by the chemosensory system (olfactory and gustatory), the auditory system and finally the visual system (Carlsen & Lickliter, 1999; Gottlieb, 1976; Hepper, 2015; Lickliter, 2000; Romagny et al., 2012; Spreen et al., 1995). Nevertheless, this chronological development of sensory systems has not yet been consistently described for every species (cuttlefish: Mezrai et al., 2019). Embryos/foetuses can perceive and possibly react to different environmental stimuli. Thus, these prenatal stimuli can influence the behaviour of individuals and their effects can persist after birth/hatching. For example, tactile and vestibular stimulations can modulate the rate of activity of young birds after hatching (domestic hens: Guyomarc'h et al., 1973). Visual stimuli, such as light, can influence the visual laterality of the young (domestic chick: Riedstra & Groothuis, 2004; Rogers, 1989, 2012; bobwhite quail: Casey & Lickliter, 1998). Many behavioural traits are modulated by the prenatal environment as: food preference (Mammals: Coureaud et al., 2002; Hepper & Wells, 2006; Hepper, 1996; Mennella et al., 2001; Birds: Bertin et al., 2010, 2012; Sneddon et al., 1998; Cuttlefish: Darmaillacq et al., 2006, 2008); maternal and social recognition (Mammals: DeCasper & Fifer, 1980; DeCasper & Spence, 1986; Graven & Browne, 2008; Hepper, 2015; Hepper, 1996; Lecanuet et al., 1987, 1987; Birds: Gottlieb, 1991; Sleigh et al., 1996) and also predator recognition (extensively studied in amphibians: Ferrari et al., 2010, 2016; Ferrari & Chivers, 2009a, 2009b, 2010; Golub, 2013; Mathis et al., 2008; Saglio & Mandrillon, 2006).

Most of the time, the effects of prenatal stimulations are beneficial for the postnatal survival of the young since perception of the environment by embryos can prepare them for their postnatal life. Nevertheless, prenatal stimulations can also have deleterious effects on behavioural profile. Since sensory systems develop with some degree of overlap, inadequate stimulation of one sensory system can reorganize the development of the others. Gottlieb, and then Lickliter and colleagues frequently demonstrated this phenomenon using environmental over-stimulation (Carlsen & Lickliter, 1999; Gottlieb et al., 1989; Honeycutt & Lickliter, 2001; Jaime & Lickliter, 2006; Lickliter, 2000, 2011; Lickliter & Lewkowicz, 1995; Sleigh & Lickliter, 1996, 1998). For example, chronic light stimulation can have an impact on maternal recognition abilities. Chicks of bobwhite quail have a preference for the maternal vocalisations

they perceived in their egg. However, when prenatal exposure to maternal calls is coupled with light stimulation, chicks do not develop this auditory preference (Honeycutt & Lickliter, 2001).

The impacts of environmental stimuli on individual postnatal phenotypes can be related to embryonic stress processes. For example, chronic prenatal exposure of rainbow trout, *Oncorhynchus mykiss*, to conspecific alarm pheromones reduces postnatal fear-related behaviour, increases activity and induces cognitive deficits (Poisson et al., 2017). These behavioural effects are similar to those reported for mammals and birds following prenatal maternal stress (Braastad, 1998; Groothuis et al., 2005; McGowan & Matthews, 2018; Sarkar et al., 2007; Weinstock, 2008). Although the effects of prenatal maternal stress are well documented in the literature, the effects on the young of stress applied to the embryo (embryonic stress) have been explored less. Indeed, stress can be experienced by non-mammalian species by both mothers and embryos when they develop in their egg and then outside their mother.

To investigate effects of direct embryonic stressful stimulations it appeared relevant to study oviparous species (Lickliter, 2005). The interest of studying them is twofold. First, as they represent the majority of species (Blackburn, 1999), it is important to understand these effects in order to protect and preserve these species. Second, because their embryos develop outside a maternal organism, their prenatal environment can be better controlled. Bird models are particularly ideal to address this question since they have been studied extensively. Bird embryos are able to perceive a wide range of environmental stimuli (Gottlieb, 1971; Höchel et al., 2002; Lalloue et al., 2003; Reynolds & Lickliter, 2002; Vince et al., 1976) and learn from them (Aigueperse et al., 2013; Bertin et al., 2010, 2012; Colombelli-Négrel et al., 2014; Gottlieb, 1991; Harshaw & Lickliter, 2010; Sleigh & Lickliter, 1996; Sneddon et al., 1998). For example, they rely on the auditory sensory modality to learn their mother's vocalisations (Gottlieb, 1991; Harshaw & Lickliter, 2010; Sleigh et al., 1996). In addition, a recent study showed that mothers' vocalisations perceived prenatally can affect long-term behaviour of young zebra finches (exposed chicks to mother heat calls were less food neophobic; Katsis et al., 2021). Bird populations can be impacted by stressful environmental changes (e.g. suboptimal temperature influenced birds behaviour and neurobiological development; Bertin et al., 2018), as well as by stressful auditory stimulations (urban sounds, human activity; reviewed in Ortega 2012) which can influence the development and the survival of young. The effects of these stressful auditory stimuli, either natural (linked to predation) or artificial (linked to human

activity) on birds remain little explored (Henriksen et al., 2011). However, authors have demonstrated that natural and artificial stresses (chronic olfactory and light stimulations) do not have the same effects on some invertebrate species, like cuttlefish (O'Brien et al., 2017). To overcome this lack of knowledge, this present study aimed to analyse the effects of repeated auditory stimulations during the prenatal period on the behavioural profile of young Japanese quail (*Coturnix coturnix japonica*). After exposing quail embryos to either natural or artificial auditory stimuli, we evaluated the impact of these stimuli on the subsequent emotional reactivity (response to experimental fear-eliciting situations) and on the social behaviour of stimulated and non-stimulated control chicks.

## Methods

### Ethics Statement

This experiment was performed in accordance with the European Communities Council Directive of 22th September 2010 (Directive 2010/63/EU) as certified by the regional ethics committee.

### Eggs treatment

#### General conditions of incubation:

The quail eggs for the current study were from a broiler line and originated from an industrial farm (Les Cailles de Chanteloup, Corps-Nuds, France). Eggs were artificially incubated in the laboratory, placed in three identical incubators (Incubator Ducat Version © TU models 140, N=61±1 eggs per incubators; the weight of the eggs was balanced between incubators). Each incubator was placed in a soundproof room to control auditory environment (mean±sd sound level in the rooms: 52.1±1.3 dB(A) and in incubators: 74.7 ±1.1 dB(A)). Egg incubation in quail typically lasts 17 days (Orcutt Jr & Orcutt, 1976). The eggs were placed for 14 days at 37.7 °C with 45% humidity and one 45° rotation every 30 minutes. Then, during 3 days, rotation was interrupted and humidity was increased to 70% in order to induce hatching.

#### Auditory stimulations during incubation:

We divided the eggs into three groups to evaluate the impact of prenatal auditory stimulation on behavioural profile: a non-stimulated control group (C); a group exposed to natural stimulations (NS) and a group exposed to artificial stimulations (AS). So that the test juveniles had a similar prenatal experience, we chose to test only chicks that hatched on the 16<sup>th</sup>, 17<sup>th</sup> and

18<sup>th</sup> days of incubation. The C group included N = 13, the NS group N = 11 and the AS group N = 23 chicks.

AS and NS embryos were exposed to prenatal stimulations from Embryonic Day 8 (ED8) to ED14, early during the development of their auditory sensory system (Höchel et al., 2002). NS embryos were subjected to vocalisations of the predatory Sparrowhawk *Accipiter nisus* (Del Hoyo et al., 1996; fundamental frequency F<sub>0</sub>=1673Hz; duration: 4.51s). AS embryos were subjected to a recording of a metal dish falling onto the floor (F<sub>0</sub>=997 Hz; 3.73s). Jones et al. (2006), studying the auditory sensory system of chicken, revealed that selectivity of responses to sound and frequency emerged around ED15 (170–4 478 Hz). Given that the incubation period of chicken lasts 21 days, we therefore assumed that the moment of auditory exposition and the stimuli frequencies used in our study would present a strong prenatal stimulation. No auditory stimulation was applied after ED14 to reduce the risk of premature hatching.

Two loudspeakers, placed directly in each incubator, glued to the centre of the right and left walls (10cm from the eggs) diffused the auditory stimuli. The loudspeakers were connected to a computer that automatically triggered the broadcast of the stimuli. The two stimuli were broadcasted at a maximum intensity of 65 dB(A) (measured on the surface of the eggs; following Alladi et al., 2002). Between ED8 and ED11, the stimuli (AS or NS depending on the group) were repeated randomly 100 times during the day (around 6 h per day for the AS and 7 h per day for the NS). To avoid habituation to these stimuli, the stimuli (AS or NS) were repeated randomly 200 times during the day (12 h per day for the AS and 14 h per day for the NS) from ED11 to ED14. For the same reason, each sequence included breaks of random intervals of one to five seconds.

### **Chicks' housing conditions**

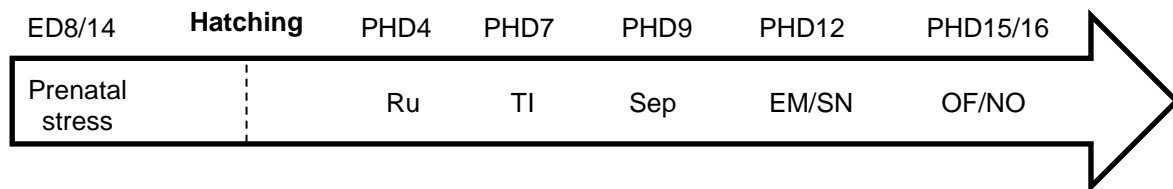
At hatching, chicks were identified individually by using coloured leg rings. Then, they were placed in experimental groups (101x65x35cm) in collective cages of approximately ten individuals each (C cage: 13 chicks; NS cage: 11 chicks; and AS cage 1: 11 chicks and AS cage 2: 12 chicks). Chicks were reared on wood shavings and provided with water and food *ad libitum*. A warming bulb was placed in each cage to ensure proper thermoregulation until the chicks were 10 days old (38±1°C in each cage). When chicks became able to regulate their own temperature, the warming bulbs were switched off and the temperature in the room was kept at 20 ± 1°C. Chicks were exposed to a 12:12h light: dark cycle.

### Somatic development:

Body mass, an indicator of somatic growth, was measured twice: at hatching (PHD0) and after the experiment on the 19<sup>th</sup> post-hatching day (PHD19) for the three experimental groups.

### Behavioural characteristics

Social behaviour and emotional reactivity were evaluated between the 4th and 16th post-hatching day (PHD4 to PHD16) by behavioural tests classically used for *C. japonica* (Forkman et al., 2007) (Fig. 1). As on previous studies done on quail, the TI test was performed on PHD7 (for example see Parois et al., 2017). Social behaviour was evaluated before emotional reactivity because the younger the chicks are, the more they are motivated to join conspecifics and less their social behaviour is biased by their emotional reactivity. Behavioural tests were carried out in a soundproof test room during the day from 9 a.m. to 6 p.m. The order passage the chicks were tested was randomized between groups. The experimenter was hidden behind a one-way glass during each behavioural test (except TI). All the chicks in each group were tested. Once the test was over, they were returned to their living cage. The behavioural variables used for analysis are described in the following paragraphs.



**Fig. 1:** Timeline of tests (ED: embryonic day; PHD: post-hatching day; Ru: runway test; TI: tonic immobility test; Sep: separation from siblings; EM: emergence test; SN: sudden noise test; OF: open field test; NO: novel object test).

### Runway (Ru):

The runway test evaluates a quail's social motivation by recording latency to join conspecifics of the same age and time spent near them (Mills et al., 1995; Suarez & Gallup, 1983). The device consisted of an opaque white plastic corridor (90x20x20cm) with a starting area at one end and a transparent cage with 4 same-aged unfamiliar chicks at the opposite (unstressed chicks raised independently of the 3 experimental groups; same chicks for each individual tested). The corridor was divided into four zones: a starting area located in the most distal zone (32cm long), an intermediate zone (32cm long), a proximate region (32cm long) and an area for contact with



the social stimulus (4cm long). To begin this test, a quail was placed in a box (18x18x18cm) at the entrance of the tunnel for 30 s. The door of the box was then opened allowing the chick free access to the device. Latency until emergence into the runway was recorded. When a subject did not emerge after 5 min, it was given the maximum score of 5 min.

#### Tonic Immobility (TI):

Tonic immobility (TI) is a natural antipredator reaction characterized by a catatonic state of the subject. Its duration is a good indicator of inherent fearfulness as TI duration is positively correlated to emotional reactivity level (Mills & Faure, 1991). To induce TI, each subject was placed on its back in a U-shaped device and held in this position for 10 s prior to release. Then, the experimenter, placed out of the subject's sight, recorded the duration of tonic immobility (with a maximum of 5 min). Instances when the subject did not remain in TI for longer than 10 s were scored 0 s.

#### Separation from siblings (Sep):

This test evaluated the responsiveness of quail placed in social isolation. To perform this test, a chick was removed from its home cage, separating it from its siblings and placed in a different room in a similar cage and left alone for 3 min. The number of distress calls and fear behaviour (pacing, jumps against the wall, running, defecation, high observation postures, and fear postures) were recorded. This test is known to relate number of calls and fear related behaviours and social separation: the greater the number of calls and fear related behaviours the higher the reactivity to social separation (Launay 1993).

#### Emergence test (EM) and sudden noise test (SN):

The emergence test evaluates willingness of individuals to leave a small dark environment, considered a safe haven, to explore an unfamiliar environment. We followed a protocol similar to that described by Mills and Faure (1986). The experimental arena is a soundproof cage with one transparent side and the floor is covered with wood chips (83x60x35cm). Quail were placed in a transport opaque box (18x18x18cm) and positioned at the entrance of the experimental arena. The transport box was kept closed for 1 min and then opened until the chick exited (maximum of 5 min). The experimenter recorded latency of emergence from the wooden box into the experimental box. When a quail had not emerged after 5 min, a maximum score of 5 min was recorded. Once the subject was in the test cage, the transport box was closed and the chick was left free in the experimental arena for 3 min. Then, a white noise was broadcast for

5 s (60dB(A) measured in the centre of the box). At the end of this white noise, the number of distress calls and fear behaviour were recorded during 3 minutes.

#### Open-field test (OF) and novel object test (NO):

In birds, the open-field and the novel object test are more commonly used to measure fearfulness and in particular to assess the fear of the novel: neophobia (Perez et al., 2020; for review see Crane & Ferrari, 2017; Greggor et al., 2015; Mettke-Hofmann 2017). To perform this test, a quail was placed at the centre of a darkened heptagonal arena marked by white walls (30cm long and 60cm high). A test lasted 5 min and began when the light was switched on. Latency of first step and frequency (number of times) of comfort behaviour (dust bath, preening and scraping the floor) were noted. A long latency to take a first step and a low frequency of comfort behaviours are considered to reflect a high emotional reactivity level (Gallup & Suarez 1980; Hawkins et al., 2001; ; Jones et al., 1992; Rushen, 2000; Zimmerman et al., 2011; for review see Jones, 1996). Indeed, it is traditionally considered that the more a bird is afraid of novelty, the less it will move. It will therefore remain motionless and silent (Gallup & Suarez 1980; Jones et al., 1992; for review see Jones, 1996).

The novel object test was performed immediately after the open field test and lasted 5 min. The light was switched off and the subject was placed at one extremity of the arena. At the same time, an unknown yellow T-shaped object (20 cm high) was placed at the opposite extremity of the device. Then the light was switched on and the latency to move close to the novel object was recorded (a maximum of 5 min was noted when a chick did not go near the object).

#### Statistical analyses

Incubation: Incubation's data did not follow a normal distribution; therefore, a non-parametric two-way ANOVA with permutation tests was used to compare incubation times between the 3 groups (R©3.6.0; Package: lmPerm; formula: p.anova; nperm=9999). Multiple pairwise comparisons were then computed (R©3.6.0; Package: RVAideMemoire; formula: chisq.multcomp; method for p-values correction: "fdr"). Chi square tests were used to compare numbers of unfertilized eggs and hatchlings in C, AS and NS groups (R©3.6.0; formula: chisq.test).

Somatic development: A Two-way ANOVA was computed to compare morpho-physiological values between the C, NS and AS chicks (R©3.6.0; Package: integrated package; formula: AOV). Tukey's Tests were then computed as a post-hoc analysis when the two-way ANOVA shows a significant difference between groups (or tendency).

**Behavioural characteristics:** In order to test the effect of prenatal stimulation on the behavioural of chicks, first we performed a principal component analysis (PCA) with Spearman correlations on the chicks behavioural variables (behavioural variables are specified above in the description of each test: latency to take the first step in the open field test; frequency of preening in the open field test; frequency of fear behaviours in the sudden noise test; latency to move close to a novel object in the novel object test; frequency of distress calls during the sudden noise test; latency of emergence of chick; duration of tonic immobility; frequency of distress calls during the separation test; frequency of fear behaviours in the separation test; latency until emergence in the runway test). We computed a varimax rotation to maximise graphical independence between the components (maximisation to the sum of the variances of the squared loadings and leaving the sub-space invariant), and we chose a criterion of PC loading of |0.5| or higher to consider that a variable was relevant to a specific component (Abdi, 2003). This PCA was executed using Excelstat® (2014). Then, in order to compare the behaviour of C, NS and AS chicks, we performed a permutation T-tests on the PCA values after Varimax rotation (R©3.6.0; Package: RVAideMemoire; formula: perm.t.test; nperm=9999).

## Results

### **Incubation durations and hatching ratio**

The average incubation durations and hatching rates did not differ significantly between the three groups (Table 1; two-way ANOVA with permutation test: group effect: df=2; mean sq=7.48; p=0.73; embryonic time effect: df=6; mean sq=39.52; p<0.001; groups\*embryonic time effect: df=12; mean sq=6.48; p=0.99). The ANOVA results show a hatching peak at ED16 for chicks in the 3 groups.

**Table 1:** number of hatchlings in relation to incubation day (from ED14 to ED20)

	<b>ED14</b>	<b>ED15</b>	<b>ED16</b>	<b>ED17</b>	<b>ED18</b>	<b>ED19</b>	<b>ED20</b>
<b>C</b>	4	1	8	2	3	0	0
<b>AS</b>	0	7	17	4	3	0	0
<b>NS</b>	0	5	7	3	3	0	1
<b>Number of hatchings:</b>	4	13	32	9	9	0	1

No significant differences were observed between the 3 groups concerning the numbers of fertilized eggs (C chicks: N=53; AS chicks: N=58; NS chicks: N=55;  $\chi^2$  test: X-squared=0.23,

df=2, p=0.89); alive hatchlings (unmalformed) (C chicks: N=18; AS chicks: N=31; NS chicks: N=19;  $\chi^2$  test: X-squared=4.62, df=2, p=0.99); and hatchlings hatched between ED16 and ED18 (C chicks: N=13; AS chicks: N=24; NS chicks: N=13;  $\chi^2$  test: X-squared=4.84, df=2, p=0.09). We had to exclude 3 chicks born with a malformed leg and limped: 1 chick in the AS group and 2 chicks in the NS group.

### **Somatic development**

At hatching (PHD0), body weight differed significantly between the three groups (Table 2: two-way ANOVA: sex effect: F=0.005; p=0.94; group\*sex effect: F=2.18 p=0.12; group effect: F=3.55 p=0.038). Posthoc tests revealed that AS chicks tended to weigh less than control chicks (posthoc TukeyHSD test: C-AS: upr=1.50; p=0.06; NS-AS: upr=1.43 p=0.15; NS-C: upr=0.78; p=0.95). On the 19<sup>th</sup> post-hatching day (PHD19) body weights no longer differed significantly between the three groups (Table 2: Two-way ANOVA: sex effect: F=0.41; p=0.53; group\*sex effect: F=0.67 p=0.52; group effect: F=0.47 p=0.63).

**Table 2:** Body weights (g) (mean  $\pm$ sd) of C, AS and NS chicks at hatching and on the 19<sup>th</sup> post-hatching day (PHD0, PHD19); # groups differ at  $0.05 \leq p \leq 0.1$  (posthoc TurkeyHSD).

<b>Body weight</b>		
	<b>PHD0</b>	<b>PHD19</b>
C chicks (6♀ and 7♂)	10.23 ( $\pm$ 0.29) #	143.85 ( $\pm$ 5.42)
AS chicks (13♀ and 10♂)	9.49 ( $\pm$ 0.18) #	139.28 ( $\pm$ 3.54)
NS chicks (2♀ and 9♂)	10.09 ( $\pm$ 0.17)	144.92 ( $\pm$ 5.15)

### **Impact on emotivity and social behaviour**

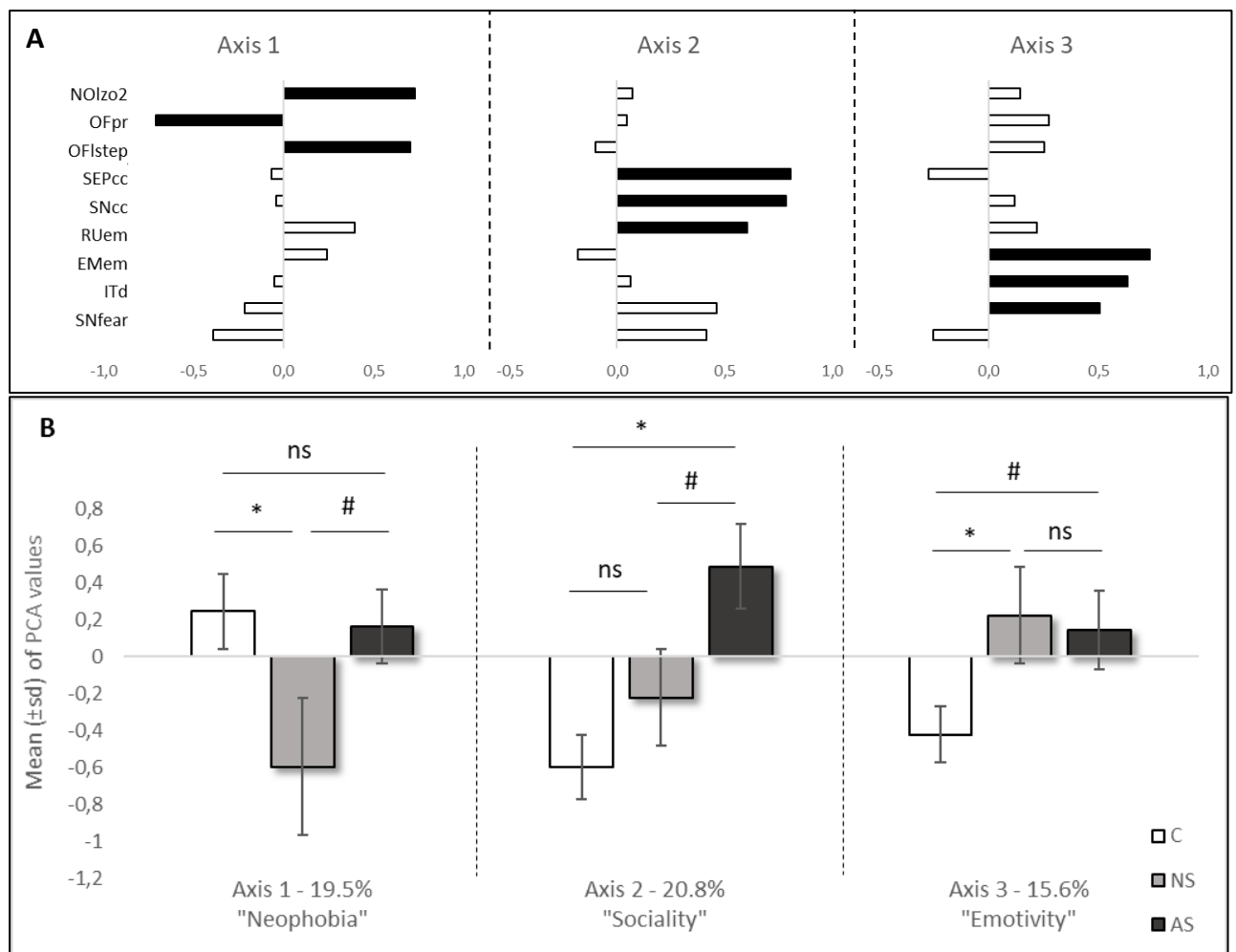
The principal component analysis identified three factors that explain 55.9% of the total variance between variables. Figures 2A and 2B present the contributions of the behavioural variables to each PCA axis (2A) and the PCA values of C, NS and AS groups for the three PCA axes (2B).

The first axis (19.5%) is explained, on one side, by long latencies to take the first step in the open field test and long latencies to approach a novel object and, on the other side, by high frequencies of preening in the open field test. This first axis reflects the level of an individual's neophobia in the presence of a novel environment or object and was named "**neophobia**" axis.

On this axis, the PCA values of C and AS chicks do not differ significantly (permutation t-:  $t=-0.27$ ;  $p=0.79$ ). However, the PCA values of NS chicks are significantly lower than those of C ( $t=-2.08$ ;  $p=0.048$ ) and tend to be lower than those of AS chicks ( $t=1.98$ ;  $p=0.055$ ). Consequently, this indicates that NS chicks started to explore quicker in novel situations than did C chicks, and are therefore considered to be less neophobic.

The second axis (20.8%) is characterized by high frequencies of distress calls of chicks when separated from their conspecifics or after hearing a sudden noise and long latencies to go towards unfamiliar conspecifics in the runway test. Thus, this axis reflects the social motivation of isolated chicks to re-establish social contact and was named "**sociality**" axis. On this axis no significant differences could be evidenced between the PCA values of C and of NS chicks ( $t=1.22$ ;  $p=0.22$ ). However, the PCA values of AS chicks are significantly higher than those of C chicks ( $t=3.37$ ;  $p=0.0014$ ; and tend to be higher than those of NS chicks  $t=1.93$ ;  $p=0.057$ ). This indicates that AS chicks appear to be more socially motivated than the control chicks.

The third axis (15.6%) is characterized by long emergence latencies in the emergence test, long durations of tonic immobility and high frequencies of fear behaviours in the sudden noise test. This last axis reflects the emotional reactivity of subjects and was named "**emotivity**" axis. On this axis, no significant differences could be evidenced between the PCA values of NS and of AS chicks ( $t=-0.22$ ;  $p=0.82$ ). However, the PCA values of C chicks are significantly lower than those of NS chicks ( $t=2.22$ ;  $p=0.038$ ) and tend to be lower than those of AS chicks  $t=1.91$ ;  $p=0.053$ ). This indicates that NS chicks express a stronger emotivity than C chicks in a fearful context.



**Fig. 2:** (A) Contributions (black bars: significant contribution) of the different behavioural variables to the three axes of the Principal Component Analysis computed on relevant behavioural data from the emotionality and sociability tests (OFIstep: latency to take the first step in the open field test; OFpr: frequency of preening in the open field test; SNfear: frequency of fear behaviours in the sudden noise test; NOLzo2 : latency to move close to a novel object in the novel object test; SNcc: frequency of distress calls during the sudden noise test; EMem; latency until the emergence of chick; ITd: duration of tonic immobility; SEPcc: frequency of distress calls during the separation test; SEPfear: frequency of fear behaviours in the separation test; RUem: latency until emergence in the runway test). (B) PCA values (mean $\pm$ sd) of the C, NS and SA groups of chicks on axis 1, 2 and 3. Permutation t-tests\*:  $p < 0.05$ ; #:  $0.05 \leq p < 0.1$ ; ns:  $p > 0.1$ . For axis 1, the more positive a value is, the more neophobic chicks will be; for axis 2, the more positive a value is, the more social reactivity chicks will have; for axis 3, the more positive a value is, the more emotional reactivity chicks will have.

## Discussion

This study assessed and compared the effect of natural and artificial stimulation on posthatching behaviours of quail. We found that chronic acoustic stimulation during the embryonic period affected quail chicks' postnatal behaviour. Moreover, these effects depended by the type of acoustic stimulation perceived by the embryos.

As showed on the PCA (3<sup>rd</sup> axis), chicks exposed to natural stimulations (predator vocalisations) were more emotive than control chicks and chicks exposed to artificial stimulations (metallic sounds) seemed to be more emotive than control chicks. This indicates that exposed chicks may react more strongly to a sudden noise, remain longer in tonic immobility and wait longer before emerging in the emergence test. Authors observed this effect when prenatal stress was applied to a mother during the laying phase (De Haas et al., 2017; Groothuis et al., 2005; Guibert et al., 2010; Henriksen et al., 2011; Houdelier et al., 2011). Indeed, maternal stress (change of diet, noise, manipulation by humans, etc.) have strong effects on birds' (quail and domestic hens) reproduction and on the postnatal behaviour of their offspring. It can induce impairment in some behaviours such as increase of emotional reactivity and activity in domestic hens (De Haas et al., 2017; Groothuis et al., 2005; Guibert et al., 2010; Henriksen et al., 2011; Houdelier et al., 2011). Prenatal maternal stress induces a modulation of hormone levels in the eggs and this in turn can influence the behaviour of chicks (variations of sex steroid levels as testosterone and androstenedione; Groothuis et al., 2005; Guibert et al., 2010; Henriksen et al., 2011; Houdelier et al., 2011). So, possibly the change in emotional behaviour we observed in this study reflects the fact that quail embryos had been stressed by the prenatal stimulations. However, we did not stress the mothers, but we did stimulate the embryos. In order to know whether stimulations perceived directly by the embryos could be considered as prenatal embryonic stress, further evidence concerning hatchlings' endocrine levels is needed to unravel this problem (i.e. hormonal dosages in eggs embryos; Henriksen et al., 2011; Quillfeldt et al., 2011; Rettenbacher et al., 2009). Finally, we can also consider the weights of the individuals in order to know if the stimuli used in our study can be stressful. Indeed, several studies reported weight loss at birth / hatching of individuals stressed during their prenatal period (mammals: Davis et al., 2009; O'Donnell et al., 2009; birds: Awerman & Romero, 2010; Schneider-Kolsky et al., 2009; Shinder et al., 2009). We found that AS chicks tended to weight less than control chicks. However, body weights at hatching were not affected

by the natural sound treatment revealing that the type of stressful stimulations could influence embryonic growth differently.

In addition to emotional reactivity, our results show that chicks' behavioural profile varied in relation to the nature of the acoustic stimulation. **On the one hand**, the PCA (2<sup>nd</sup> axis) showed that AS chicks were more social than controls: they seemed to be more responsive to social isolation. In birds, breaking the links between social partners induces distress responses as distress calls and jumping (Schweitzer et al., 2010). In the present study, AS individuals were more responsive to social separation, emitting more distress calls than C individuals. However, the 2<sup>nd</sup> axis showed also that AS chicks appeared less motivated to go towards unfamiliar chicks: their latency to go towards unknown chicks in the runway test tended to be longer. These two results appear contradictory because individuals more vulnerable to social isolation usually have greater social motivation to go towards congeners (Recoquillay et al., 2013; Richard et al., 2008). Nevertheless, possibly, chicks are more motivated to go towards familiar chicks than towards unknown chicks. We therefore hypothesize that AS quail were more attached to familiar partners than were unfamiliar chicks, suggesting that the artificial prenatal stimulations had influenced the development of their social behaviour. Aigueperse et al.'s study (2020) presented similar results related to maternal prenatal stress. Chicks of mothers stressed during the laying phase (social stress) and thereafter adopted by unstressed mothers, emitted more requests to their mother (more distress calls and fear behaviours when they were isolated from their mother). **On the other hand**, the PCA (1<sup>st</sup> axis) showed that prenatal stimulations had effects on neophobia: NS chicks seemed to be less neophobic than C chicks in an open-field situation (shorter first step latency and they had higher rates of preening) and in the presence of a novel object (they approached unknown objects much faster). Short latencies to take the first step were considered to reflect high exploration tendency (Rushen, 2000) and high frequencies of preening to reflect low emotional levels (Hawkins et al., 2001; Rushen, 2000; Zimmerman et al., 2011). These results indicate that in anxiety-inducing conditions, NS chicks are less neophobic, more inclined to explore and approach a novel object. Therefore, natural prenatal stimuli could play a key role in shaping an adaptive response to a stressful environment. Indeed, in the case of chronic stress, most responses are adaptive and benefit the survival of the animal (reviewed in Herman, 2013). For example, predator cues are important for amphibian embryos since they enhance their short-term survival after hatching when predators are present (Ferrari et al., 2010; Ferrari & Chivers, 2010; Mathis et al., 2008).



In the present study, chicks' behavioural profile differed depending on the type of acoustic stimulation they had perceived. Whereas artificial stimulations affected social behaviour, natural stimulations influenced neophobia. In the literature, artificial sounds such as traffic sounds probably have the most widespread and greatest indirect effect on birds (reviewed in Kociolek et al., 2011; Reijnen et al., 1996). Birds may be particularly affected by anthropogenic sounds because they rely extensively on acoustic communication (reviewed in Kociolek et al., 2011). Conversely, natural stimulations as predator cues are important since they can prepare individuals for their future living conditions. Many oviparous species are able to detect predator cues before hatching (for example in cephalopods: Mezrai et al., 2020; amphibians: Ferrari et al., 2010; Ferrari & Chivers, 2010; Mathis et al., 2008; and birds: Noguera & Velando, 2019). These stimulated embryos presented a developmental plasticity of their defensive phenotypes that enhanced their short-term survival rate after birth when predators are present (Ferrari et al., 2010; Ferrari & Chivers, 2010; Mathis et al., 2008; Noguera & Velando, 2019). These differential effects show that the impacts of prenatal auditory stimulations depend on characteristics of the stimulus. Two hypotheses could explain this: (1) the structure of the sound could play a role in these prenatal effects. Indeed, although we controlled the sound level of the two stimuli, they still differed in frequency, duration and sequential organization, and these parameters may be perceived at different times during embryonic development (Konishi, 1973). (2) Our two stimuli may also be "integrated/interpreted" differently by each individual, inducing distinct behavioural profile. This phenomenon is more difficult to demonstrate. It would however be instructive to find out whether there are other embryonic responses to both stimuli including physiological and behavioural markers. For example, cardiac responses, movements or vocal responses of embryos could be evaluated to determine whether they differ following the AS and NS stimuli. Data concerning these traits would help to decide between these two hypotheses. To sum up, over-stimulation caused by natural and artificial stimuli could possibly have over-stimulated embryos' auditory system. The functions of these prenatal stimuli have long been studied by Gottlieb who identified three potential functions of early prenatal experiences: "maintenance"; "induction" and "facilitation" (Gottlieb, 1981; 1971; 1976a; Gottlieb, 2003). Stimulations can have a "maintenance" effect that helps maintain the integrity of an already fully-formed neural or behavioural system (Gottlieb, 1976a). These stimuli can also have an "inducing" effect, directing the development in one direction rather than another. Finally, prenatal stimulation can "facilitate" behavioural development. Gottlieb described facilitation as a process leading to accelerated behavioural development. Behavioural capacity appears earlier in stimulated individuals than in non-stimulated individuals. Unlike

induction, facilitation experiences regulate maturation, improve performance, and increase perceptual differentiation and learning abilities (Gottlieb, 1976b; Gottlieb, 1971). In our study, the prenatal stimulations possibly directed the chicks' behavioural profile in one direction rather than another (inductive effect).

To conclude, despite the small number of individuals studied, we identified different behavioural profiles and these profiles varied according to the type of auditory stimulation perceived. On the one hand, artificial stimulations, linked to an anthropic environment will make individuals more emotional and more social. On the other hand, natural stimuli, linked to predation pressure, will make chicks more responsive to emotional events but also to explore more in an unfamiliar environment. Auditory stimuli therefore have an impact on individual's behaviour profile. They can induce either positive or negative adaptation effects. Study of the effects of these environmental stimuli therefore becomes essential because organisms (terrestrial and marine) constantly undergo variations of their environment to which they must provide appropriate individual responses. These responses can affect their growth, reproduction and fertility and thus have consequences on the spatiotemporal dynamics of the populations to which they belong.

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## **Competing interests**

The authors declare no competing interests.

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## **Data availability**

The data that support the findings of this study are available on request from the corresponding author: S. Lumineau.

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